

Fluorescence Spectroscopy of the Cervix: Influence of Acetic Acid, Cervical Mucus, and Vaginal Medications

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Background and Objective: Fluorescence spectroscopy has been shown to provide information useful in the detection of cervical dysplasia. The goal of this study was to determine if substances found on the cervix such as acetic acid, mucus, and vaginal medications can influence the fluorescence in the spectral region useful for discriminating normal cervical tissue from abnormal tissue.

Study Design/Materials and Methods: Fluorescence spectra were collected at 337 nm excitation from the cervix in vivo both before and after application of acetic acid; the data were analyzed to identify the effects of the acetic acid on the spectra. Cervical mucus was acquired from patients referred for colposcopy and frozen until measurements were taken. Fluorescence excitation-emission matrices (EEMs) were measured for the mucus samples. Additionally, the transmission spectra of mucus were measured to determine if its absorption could influence the fluorescence signal measured from the tissue. EEMs were measured for samples of commonly prescribed vaginal medications. All EEMs were compared to those of cervical biopsies.

Results: Acetic acid introduces changes in both the lineshape and intensity of the spectra. On average, the changes are more significant in spectra of abnormal tissue. Cervical mucus was found to have no significant absorption bands, but the measured fluorescence was approximately the same order of magnitude as that measured from the cervix in vitro. Most medications exhibited significant fluorescence in the spectral region of diagnostic interest for the cervix.

Conclusions: Acetic acid appears to increase the differences in fluorescence emission spectra of normal and pre-cancerous cervical tissues; thus, its use is beneficial. The presence of cervical mucus can possibly interfere with the collection of fluorescence spectra for tissue classification. Patients should not use vaginal

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INTRODUCTION

Fluorescence spectroscopy has been demonstrated to be an effective technique for detecting malignancies in a number of organ sites [1-8]. Several groups have applied fluorescence spectroscopy for the detection of cervical dysplasia [5-8]. Our research group has been developing diagnostic devices based on fluorescence spectroscopy to diagnose squamous intraepithelial lesions (SILs) of the cervix in vivo [7,8]. A multivariate statistical algorithm has been developed and evaluated, which can differentially diagnose SILs based on fluorescence emission spectra collected at three excitation wavelengths (337, 380, and 460 nm) [8]. The diagnostic performance of this algorithm could be influenced, either positively or negatively, by exogenous or endogenous agents present on the cervix. For this reason, we now explore the spectroscopic properties of several substances commonly found on the cervix: acetic acid, cervical mucus, and vaginal medications.

Acetic acid is routinely used during colposcopy, a procedure involving examination of the cervix in situ with a low power microscope, to enhance visual differences between normal and abnormal regions of the cervical epithelium. Areas which may develop into cervical cancer undergo a transient whitening (acetowhitening) visible to the naked eye. The previously mentioned diagnostic algorithm has been developed based on fluorescence spectra collected from the cervix after the application of acetic acid. However, the effect of the acetic acid on the measured tissue spectra has not been investigated. This study was designed in part to determine the extent of the differences between spectra collected before and after acetic acid is applied, and determine whether or not the changes induced by acetic acid are beneficial or necessary for fluorescence-based diagnosis.

Cervical mucus is often present on the ectocervix, as well as in the folds of the endocervical canal. Mucus production and composition varies during the menstrual cycle. During the proliferative phase it can be quite viscous and optically turbid, whereas during ovulation it is a clear transparent fluid. During colposcopy, attempts

are made to remove this mucus from the cervix to improve visualization of the tissue, but complete removal can be difficult. Therefore, a second goal of this study was to determine if the presence of cervical mucus is likely to interfere with the performance of our diagnostic algorithms, either by absorbing excitation or fluorescent light or by producing its own fluorescence.

Vaginal medications are commonly prescribed for the treatment of yeast and other infections. When these medications are applied to the vagina, a residue can be left on the cervix. For this reason, another goal of this study was to measure the fluorescence of different medications to determine if they fluoresce in the spectral region of diagnostic interest.

The results of this study show that acetic acid introduces changes in both the lineshape and intensity of fluorescence spectra of the cervix. Because these changes tended to be more extreme for abnormal tissue, acetic acid appears to be beneficial when applying spectroscopic techniques for detecting SILs. Cervical mucus was found to have minimal absorption and fluorescence intensities, which were approximately the same order of magnitude as those of cervical biopsies. Many vaginal medications also produce strong fluorescence, which could interfere with the spectra measured from the cervix.

MATERIALS AND METHODS

Acetic Acid

We measured fluorescence spectra of the cervix in vivo from 115 patients referred for colposcopy and treatment due to the presence of cervical dysplasia. Informed consent was obtained and the study was reviewed and approved by the Institutional Review Board at the University of Texas MD Anderson Cancer Center and the University of Texas at Austin. The spectra were acquired using a specialized multi-pixel fiber optic probe (Fig. 1) with the system shown in Figure 2.

The probe consists of 62 fibers with 200 μm core diameter, arranged as 31 fiber pairs at the distal end. Each fiber pair defines a pixel on the tissue, since one fiber of each pair is used for de-

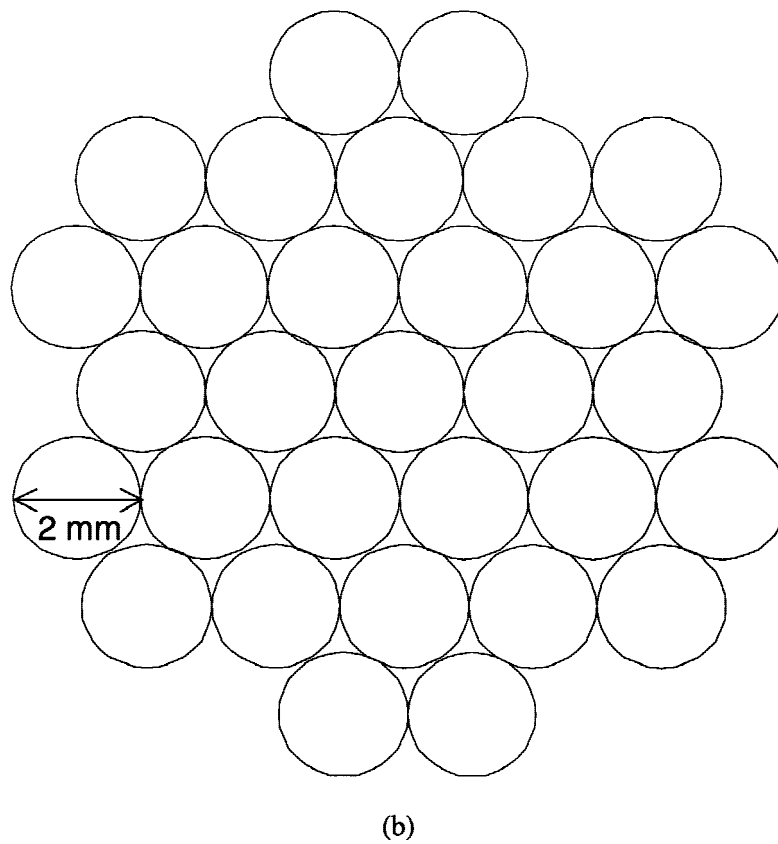
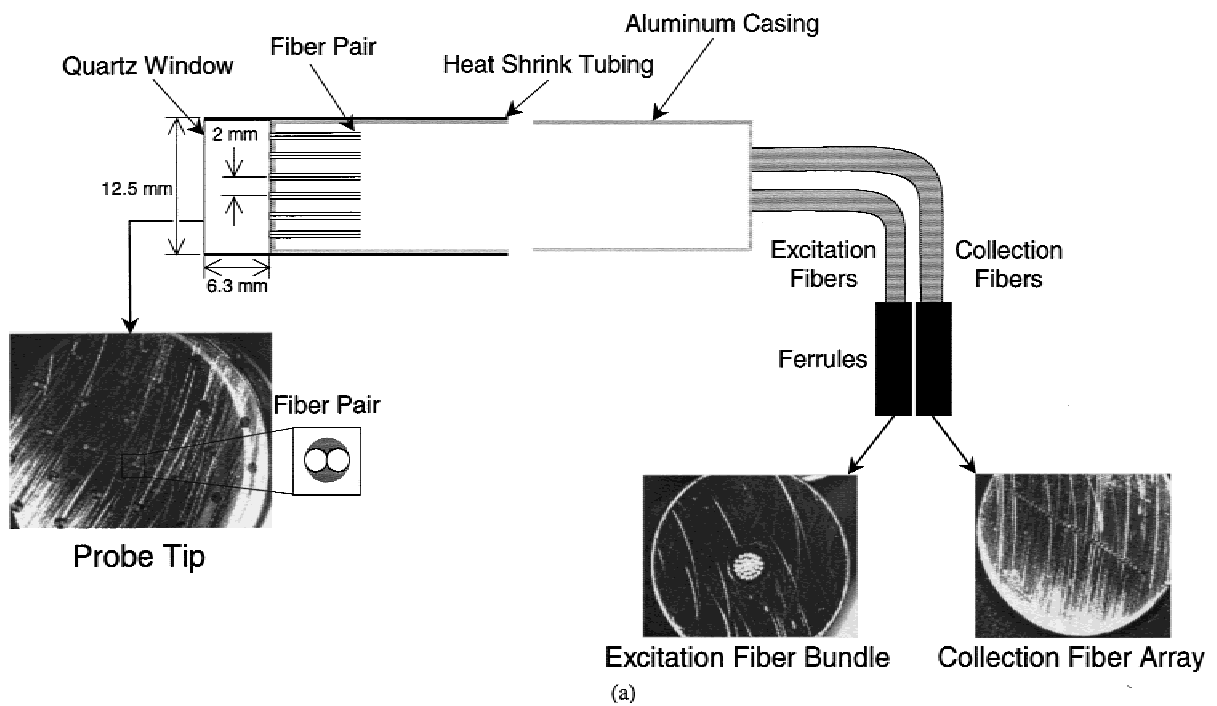


Fig. 1. Multi-pixel fiber optic probe. **a:** Probe design. **b:** Pixel arrangement on tissue.

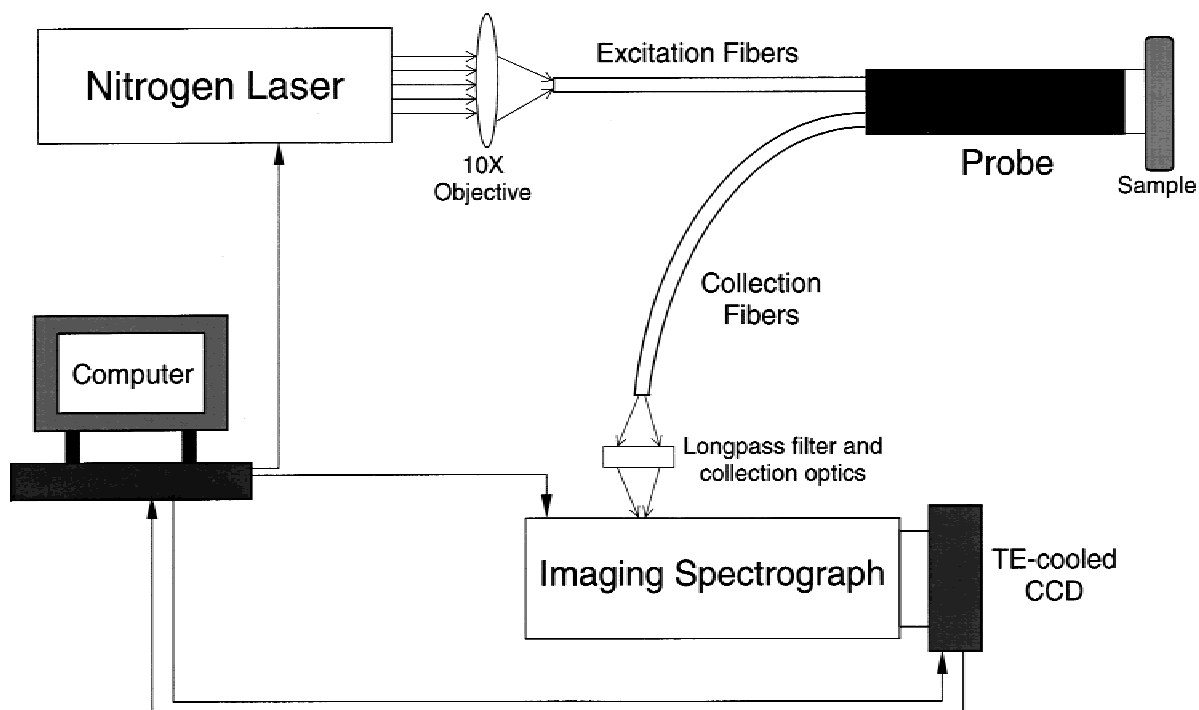


Fig. 2. System used for in vivo acquisition of fluorescence spectra from the cervix.

livering excitation light to the tissue and the other fiber is used for collecting fluorescence from the area illuminated by its corresponding excitation fiber. The fiber pairs, each separated from adjacent pairs by 2 mm, are arranged in a hexagonal pattern such that, at a fixed distance from the probe, there are closely packed, non-overlapping 2 mm diameter pixels. A quartz window is affixed to the front of the probe to maintain a fixed distance between the probe and the tissue.

The system employs a nitrogen laser (LN 1000, Laser Photonics, Orlando, FL), providing 337 nm excitation, whose light is coupled into the excitation fibers of the probe via a 10× microscope objective. At the tissue, the pulse energy was approximately 0.4 mJ, spread out over the 1.25 cm diameter area. Fluorescence was integrated from 60 pulses of the source. Light from the collection fibers is passed through a longpass filter to reject the Rayleigh scattered excitation light and then focused onto the slit of an imaging spectrograph (250IS, Chromex Inc., Albuquerque, NM). The spectrum from each collection fiber is imaged onto a TE-cooled CCD array (TE/CCD-576 EUV, Princeton Instruments, Trenton, NJ). The entire system is under computer control.

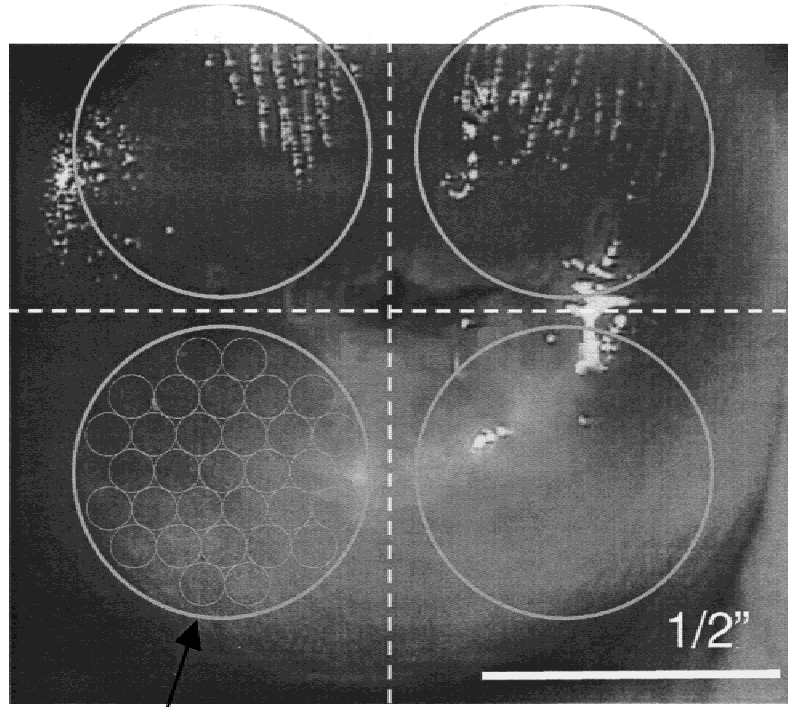
To correct for the non-uniform spectral response of the detection system, correction factors were obtained by measuring the spectrum of an NIST traceable tungsten ribbon filament lamp

(550C, Optronic Laboratories Inc., Orlando, FL). The spectra measured from each patient were intensity calibrated by measuring the fluorescence of an FAD standard solution prior to fluorescence measurements of the tissue.

As shown in Figure 3, spectra were acquired by placing the probe in each of the four quadrants of the cervix (12–3 o'clock, 3–6 o'clock, 6–9 o'clock, 9–12 o'clock), both before and after 6% acetic acid was applied to the cervix for colposcopy. The patients were then treated by the loop electrosurgical excision procedure to remove the dysplastic region of tissue, and the pathology of the excised specimen was obtained. The pathology results were reported by the clock position of each section of the specimen. The possible classifications included normal squamous mucosa, inflammation, low grade SIL or high grade SIL.

Due to fixation-induced shrinkage and specimen processing, we were unable to correlate the spectra from individual pixels with a precise histopathologic diagnosis. Therefore, only those spectra that were acquired from an entirely normal (non-SIL) or entirely abnormal (SIL) quadrant were included in this study. There were 73 patients who had at least one quadrant that satisfied this criterion.

For each patient, the spectra were divided into four categories: normal squamous mucosa before and after acetic acid (NL/Bef, NL/Aft), abnor-



Approximate Probe Position

Fig. 3. Probe placement method on the cervix.

mal, including inflammation LGSIL or HGSIL, before and after acetic acid (ABNL/Bef, ABNL/Aft).

Data were analyzed to determine (1) how much change in cervical fluorescence was typically induced by acetic acid for a given type of tissue (i.e., normal or abnormal) and (2) how much the contrast between normal tissue fluorescence and abnormal tissue fluorescence could be enhanced by acetic acid.

Data from each patient were used to calculate ratios of the average spectrum from one category to the average spectrum from another category:

$$X/Y(\lambda_m) = \frac{\sum_{i=1}^{N_X} x_i(\lambda_m)}{N_X} \cdot \frac{N_Y}{\sum_{j=1}^{N_Y} y_j(\lambda_m)},$$

where $X/Y(\lambda_m)$ is the fluorescence intensity ratio of category X to category Y at emission wavelength λ_m , N_X is the number of spectra in category X for the patient, $x_i(\lambda_m)$ is the fluorescence intensity at symbol λ_m from spectrum i of category X, N_Y is the number of spectra in category Y for the patient, and $y_j(\lambda_m)$ is the fluorescence intensity at λ_m from spectrum j of category Y. We calculated the ratios for four combinations of categories:

$$\frac{NL/Bef}{NL/Aft}, \frac{ABNL/Bef}{ABNL/Aft}, \frac{NL/Bef}{ABNL/Bef}, \text{ and } \frac{NL/Aft}{ABNL/Aft}.$$

The first two ratios provide insight into how much the fluorescence of a particular type of tissue is affected by acetic acid. Comparing the second two ratios indicates how much the contrast between normal tissue fluorescence and abnormal tissue fluorescence is affected by acetic acid. Some patients had the same pathology for all four quadrants, so these patients had data in only two of the four categories.

Ratios from 56 and 48 patients were then used to calculate average values of

$$\frac{NL/Bef}{NL/Aft} \text{ and } \frac{ABNL/Bef}{ABNL/Aft},$$

respectively. The number of patients used for each of these averages was based on the number of patients that had at least one quadrant with the given classification (normal or abnormal). Ratios from 32 patients were used to calculate average values of

$$\frac{NL/Bef}{ABNL/Bef} \text{ and } \frac{NL/Aft}{ABNL/Aft}.$$

For these averages, only those patients who had both normal and abnormal quadrants were included in the calculations.

Another method of quantitatively estimating the effect of acetic acid on the fluorescence data from each patient was by comparing one category's distribution of fluorescence intensities at a given emission wavelength vs. that of another category. By performing this comparison at each emission wavelength, we could determine the wavelengths at which the fluorescence intensities changed significantly between categories. Because the intensity distributions tended to be distributed non-normally, we used the Mann-Whitney test [9], a non-parametric statistical test, to compare distributions. We were specifically concerned with two comparisons: NL/Bef vs. ABNL/Bef and NL/Aft vs. ABNL/Aft. These comparisons provide insight into how much the contrast between normal and abnormal tissue fluorescence is enhanced by acetic acid. Data from the same 32 patients as above were used to determine what percentage of patients demonstrated statistically significant changes in fluorescence intensity distributions of the two categories at each emission wavelength, both before and after application of acetic acid.

Cervical Mucus

Samples of cervical mucus were collected from the cervical os from three colposcopy patients and were frozen until used in this study. These samples, approximately 0.5 ml in volume, were thawed and diluted in 3 ml of normal buffered saline prior to transmission and fluorescence measurements. The dilution was necessary to obtain a sufficient volume for containment in a 1 cm pathlength cuvette. Transmission spectra of the diluted samples were measured with an absorption spectrophotometer (U-3300, Hitachi, Tokyo,

Japan). The wavelength range was 250–700 nm in 1 nm increments. Fluorescence excitation-emission matrices (EEMs) were measured of the diluted samples using a standard scanning spectrofluorimeter (Fluorolog II, SPEX, Edison, NJ). A front face excitation-collection geometry, whereupon the illumination beam is focused at the front surface of the sample and the fluorescence is collected at a small angle ($\approx 20^\circ$) with respect to the illumination, was used for the fluorescence measurements. This geometry mimics that which is used by a fiber optic probe for in vivo fluorescence measurements. Excitation wavelengths ranged from 250 to 550 nm in 10 nm increments, and the emission spectra were collected in 5 nm increments. The EEMs were corrected for variations in the intensity of the excitation source using a Rhodamine B quantum counter and corrected for the non-uniform response of the collection system using correction factors supplied with the instrument. Intensities are reported in the same set of arbitrary units for all samples measured with this instrument.

Vaginal Medications

A survey asking the most frequently prescribed vaginal medications was mailed to 18 faculty and 23 residents in Obstetrics, Gynecology, and Reproductive Sciences at the University of Texas Health Science Center and nine faculty and six fellows in Gynecologic Oncology at the University of Texas M. D. Anderson Cancer Center. Thirty one of these physicians responded to the survey (53% response ratio). Based on these responses, we compiled a list of the 16 most frequently used medications to include in our analysis (Table 1).

Using a similar approach to that used for the cervical mucus samples, an EEM was measured using the same scanning spectrofluorimeter for an undiluted sample of each medication in the list. For these EEMs, excitation wavelengths ranged from 250 to 550 nm in 20 nm increments, with emission spectra recorded in 5 nm increments. The data were corrected in the same manner as the mucus data and are reported in the same set of arbitrary units.

RESULTS

Acetic Acid

Figure 4 shows an example of spectra from a normal site and an abnormal site from one patient.

TABLE 1. The Common Usage and the Frequency of Mention in the Physician Survey for Each Medication

Sample #	Name	Manufacturer	Usage	Freq. [%]
1	Premarin®	Ayerst Lab. Inc.	Hormone replacement	39
2	Monistat® 7	Ortho Pharm.	Rx* yeast	52
3	Efudex®	Roche Lab.	Rx VAIN	3
4	Aci-Jel	Ortho Pharm.	Healing	3
5	Sultrin™	Ortho Pharm.	Rx infection	6
6	Vagistat®-1	Bristol-Myers Squibb	Rx yeast	6
7	Terazol® 3	Ortho Pharm.	Rx yeast	48
8	Thermazene®	Sherwood Medical	Rx infection	3
9	Cleocin®	Upjohn	Rx infection	52
10	Femstat®	Syntex Lab.	Rx yeast	3
11	Metrogel®	Curatek Pharm.	Rx infection	29
12	AVC™ cream	Marion Merrell Dow	Rx infection	3
13	Replens®	Parke-Davis	Vag. lubricant	6
14	Terazol® 7	Ortho Pharm.	Rx yeast	48
15	Trysul®	Savage Lab.	Rx infection	
16	Nystatin Vag. Tablets	Rugby Lab.	Rx chronic yeast infections	3

*Rx = treatment.

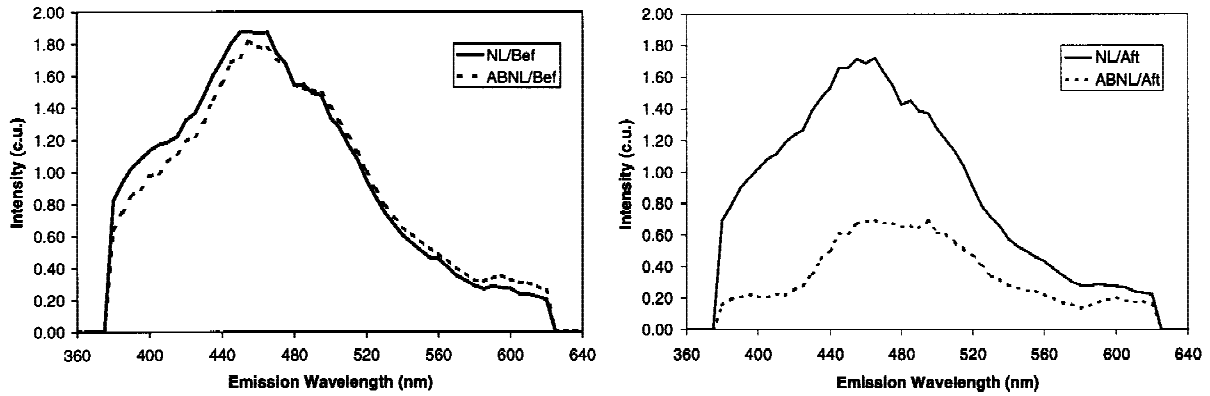


Fig. 4. Fluorescence spectra before and after acetic acid from normal and abnormal sites.

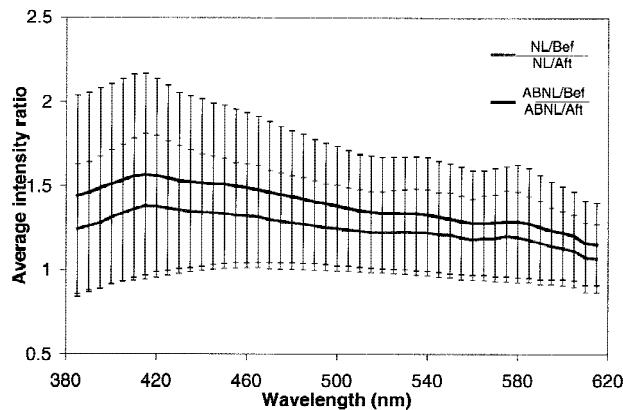


Fig. 5. Average values \pm standard deviation of $\frac{NL/Bef}{NL/Aft}$ and $\frac{ABNL/Bef}{ABNL/Aft}$ ratios.

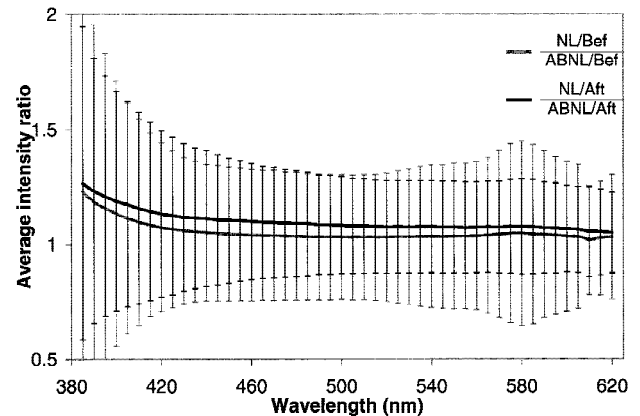


Fig. 6. Average values \pm standard deviation of $\frac{NL/Bef}{ABNL/Bef}$ and $\frac{NL/Aft}{ABNL/Aft}$ ratios.

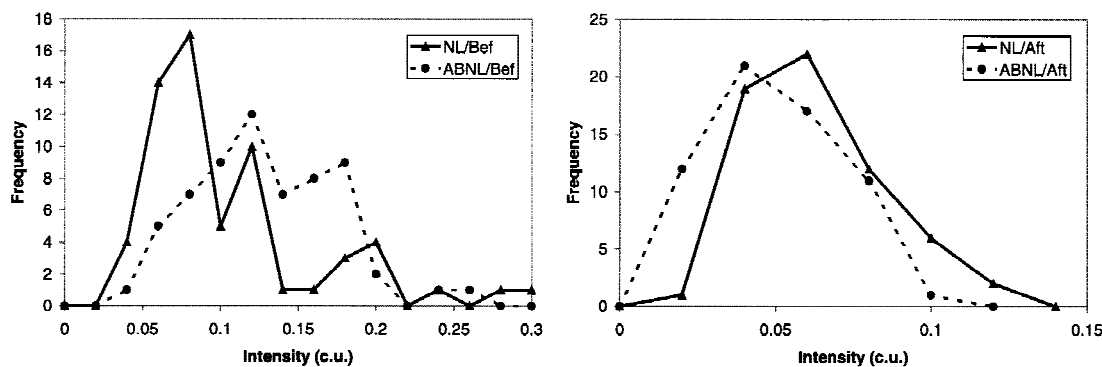


Fig. 7. Distributions of fluorescence intensities at 460 nm emission from one patient.

As can be seen from this figure, there is only a slight difference between the normal and abnormal spectra before acetic acid. When acetic acid is applied, the peak fluorescence intensity from the same abnormal site is much less than that of the same normal site, and the lineshape is also changed.

Plots of the ratios

$$\frac{NL/Bef}{NL/Aft} \text{ and } \frac{ABNL/Bef}{ABNL/Aft}$$

as a function of emission wavelength averaged over all patients, are shown in Figure 5. The error bars indicate the standard deviations. Although the variance is high, the ratios from both types of tissue are always greater than unity at all emission wavelengths. This indicates that acetic acid typically causes a reduction in the measured fluorescence. This reduction tends to be greater for abnormal tissue, since its ratio is greater than that of normal tissue. Additionally, it is interesting to note that the peaks in the ratio averages (420, 540, 580 nm) correspond to the location of absorption bands of hemoglobin.

The ratios

$$\frac{NL/Bef}{ABNL/Bef} \text{ and } \frac{NL/Aft}{ABNL/Aft}$$

averaged over all patients are shown in Figure 6 as a function of wavelength. Both ratios are greater than one on average at all emission wavelengths, indicating that the fluorescence intensity of abnormal tissue typically is less than that of normal tissue. Again the variance is high, but on average acetic acid induces a greater relative intensity difference between normal and abnormal tissue. After acetic acid, variance in the average ratio of the fluorescence intensity of normal tissue

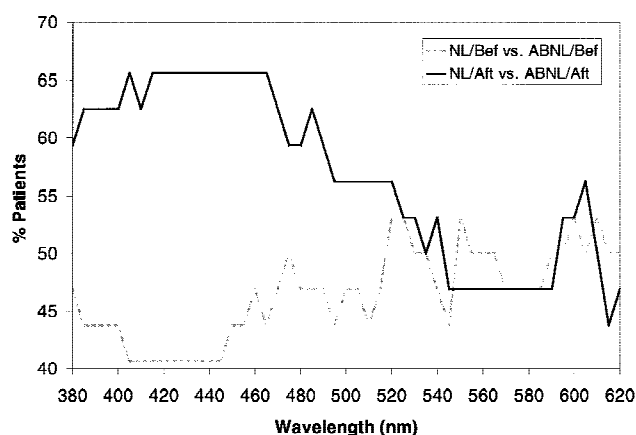


Fig. 8. Percentage of patients for which intensity distributions between categories was significantly different at each emission wavelength.

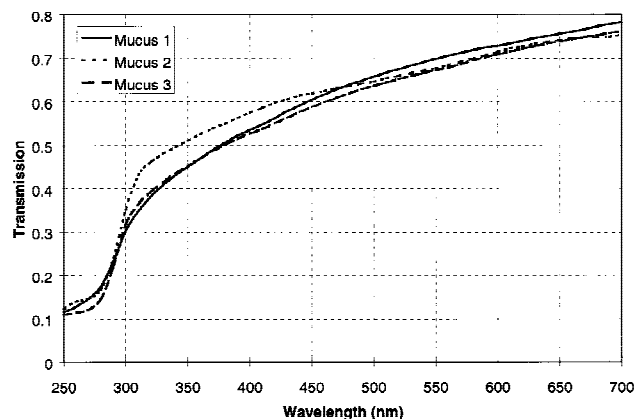


Fig. 9. Transmission spectra of mucus samples.

to that of abnormal tissue is less, a characteristic that indicates that acetic acid tends to produce a more consistent ratio of the fluorescence intensity between normal and abnormal tissue.

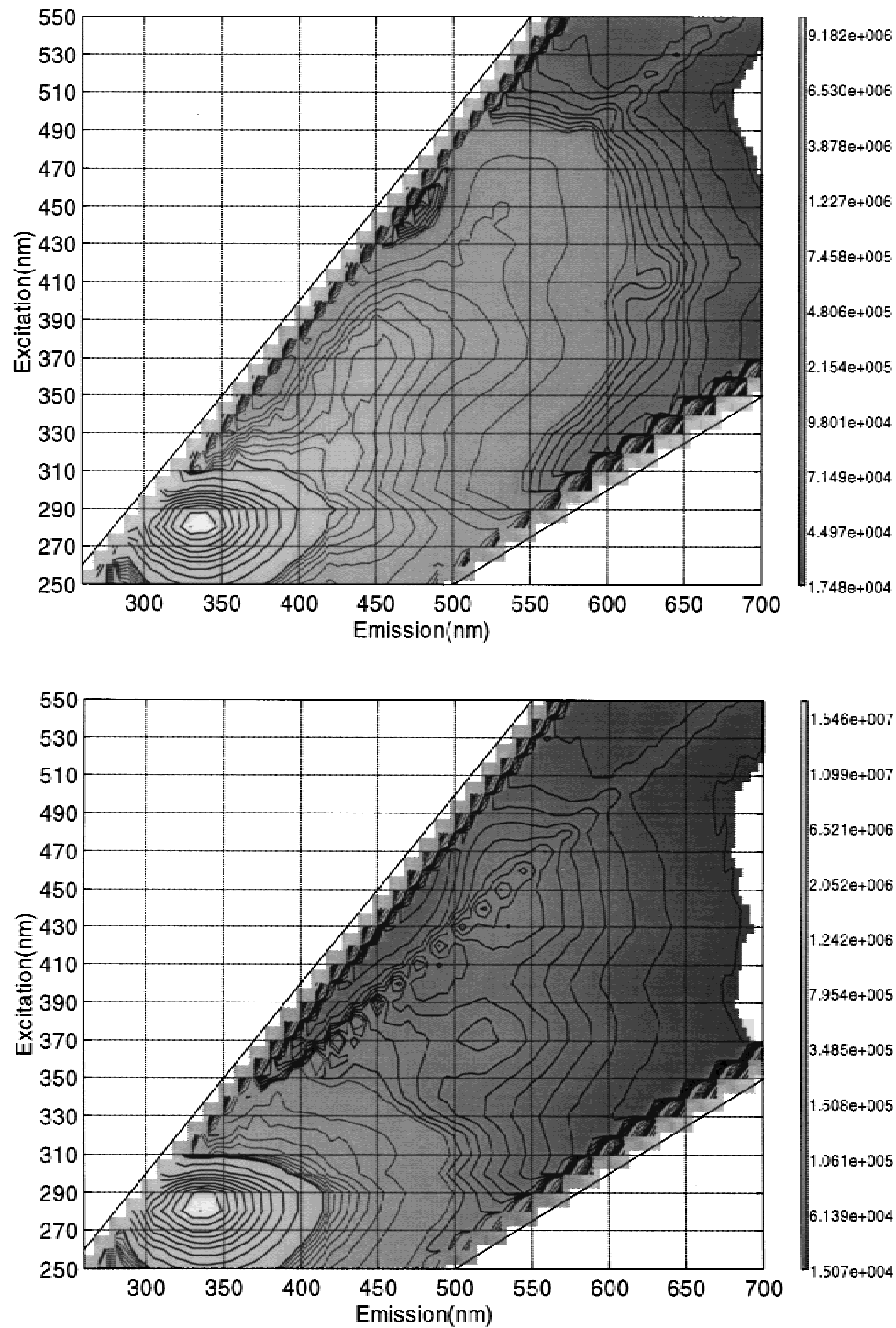


Fig. 10. EEMs of two mucus samples.

Figure 7 shows an example of the intensity distributions at 460 nm emission from one patient for normal and abnormal tissues before and after acetic acid. In this example, it can be seen that after acetic acid the distributions appear more well-defined and distinct. Figure 8 shows the per-

centage of patients for which the intensity distributions of the two categories under comparison are significantly different at each emission wavelength. The significance level (α) is 0.05. At wavelengths below 500 nm, acetic acid clearly causes a greater percentage of patients to exhibit differ-

TABLE 2. Excitation-emission Peak Locations and Intensities for Mucus Samples, Vaginal Medications, and Cervical Biopsies*

Sample	Ex. peak (nm)	Em. peak (nm)	Intensity (c.u.)
Mucus 1	280	340	9.6 E6
	350	450	7.0 E5
	450	535	2.6 E5
Mucus 2	280	340	2.2 E7
	350	450	1.8 E5
	450	535	7.2 E4
Mucus 3	280	340	1.6 E7
	350	450	1.5 E5
	450	535	1.1 E5
Medication 1	310	350	4.9 E7
	370	470	2.9 E7
Medication 2	—	—	—
Medication 3	—	—	—
Medication 4	390	550	2.1 E7
Medication 5	370	450–455	1.8 E7 (B)
Medication 6	330	385	3.2 E8
Medication 7	310	360	4.8 E8
Medication 8	390	430	6.4 E7
Medication 9	310	350–355	2.1 E7 (B)
Medication 10	—	—	—
Medication 11	310	350	1.6 E6
Medication 12	310	350	4.4 E7
Medication 13	310	350	6.6 E6
Medication 14	310	360	4.1 E8
Medication 15	370	455	1.7 E7 (B)
Medication 16	310	440–445	2.2 E7
NL Biopsy	290	330	5.3 E7
	350	450	3.6 E6
	450	535	1.7 E6
ABNL Biopsy	290	330	2.5 E7
	350	450	7.1 E5
	450	535	4.2 E5

*B = broad peak.

ences between normal and abnormal tissue in their fluorescence spectra. At emission wavelengths above 500 nm, acetic acid appears to provide no appreciable enhancement of intensity differences in the spectra.

Cervical Mucus

The transmission of mucus was examined to see if there were any strong absorption bands that may significantly affect the lineshape of fluorescence spectra collected from the cervix with mucus present. The measured mucus transmission spectra are shown in Figure 9. Mucus demonstrates strong protein absorption below 300 nm. In general, no significant absorption bands were observed in the visible part of the spectrum.

Representative fluorescence EEMs for two samples of mucus are shown in Figure 10. The

mucus fluorescence is strongest at the tryptophan emission (280/340), and small intensities can be observed at NADH (350/450) and FAD (450/535) emission peaks. The excitation-emission maxima locations and intensities in arbitrary units for each of the mucus samples are listed in Table 2.

Vaginal Medications

EEMs of the two most commonly cited vaginal medications, Premarin and Monistat, are shown in Figure 11, and the peak excitation-emission pairs and corresponding intensities (in arbitrary units) for all the medications are found in Table 2. In examining the EEMs of the 16 medications, 10 of them have clear fluorescent peaks whereas six of the agents exhibit very broad fluorescent peaks or no recognizable peak at all. As can be seen in Table 2, of the 10 medications with strong peaks, only Premarin has more than one significant peak, and eight of the medications have peaks at 310 nm excitation.

DISCUSSION

Acetic Acid

The results of both the Mann-Whitney test and the ratio calculations suggest that applying acetic acid to the cervix before measuring 337 nm excited fluorescence enhances the discrimination capability. The diagnostic algorithm developed by our group, based on fluorescence spectra acquired at 337, 380, and 460 nm excitation after the application of acetic acid, was able to achieve 82% sensitivity and 68% specificity when discriminating SILs from non-SILs, and 79% sensitivity and 78% specificity when discriminating low-grade SILs from high-grade SILs [8]. It appears that acetic acid contributed to the good performance of this algorithm.

Since only 337 nm excitation was used for the spectra collected in this study, additional data is needed to determine the effect of acetic acid-induced changes in spectra collected at the other two diagnostically useful excitation wavelengths: 380 nm and 460 nm. Further work is also needed to determine the concentration dependence and time course of acetic acid-induced changes in fluorescence spectra. Since acetowhitening is a transient effect, lasting typically several minutes, the time at which fluorescence is measured after acetic acid application would be a significant clinical study parameter. This parameter was not closely monitored in this study and therefore could have

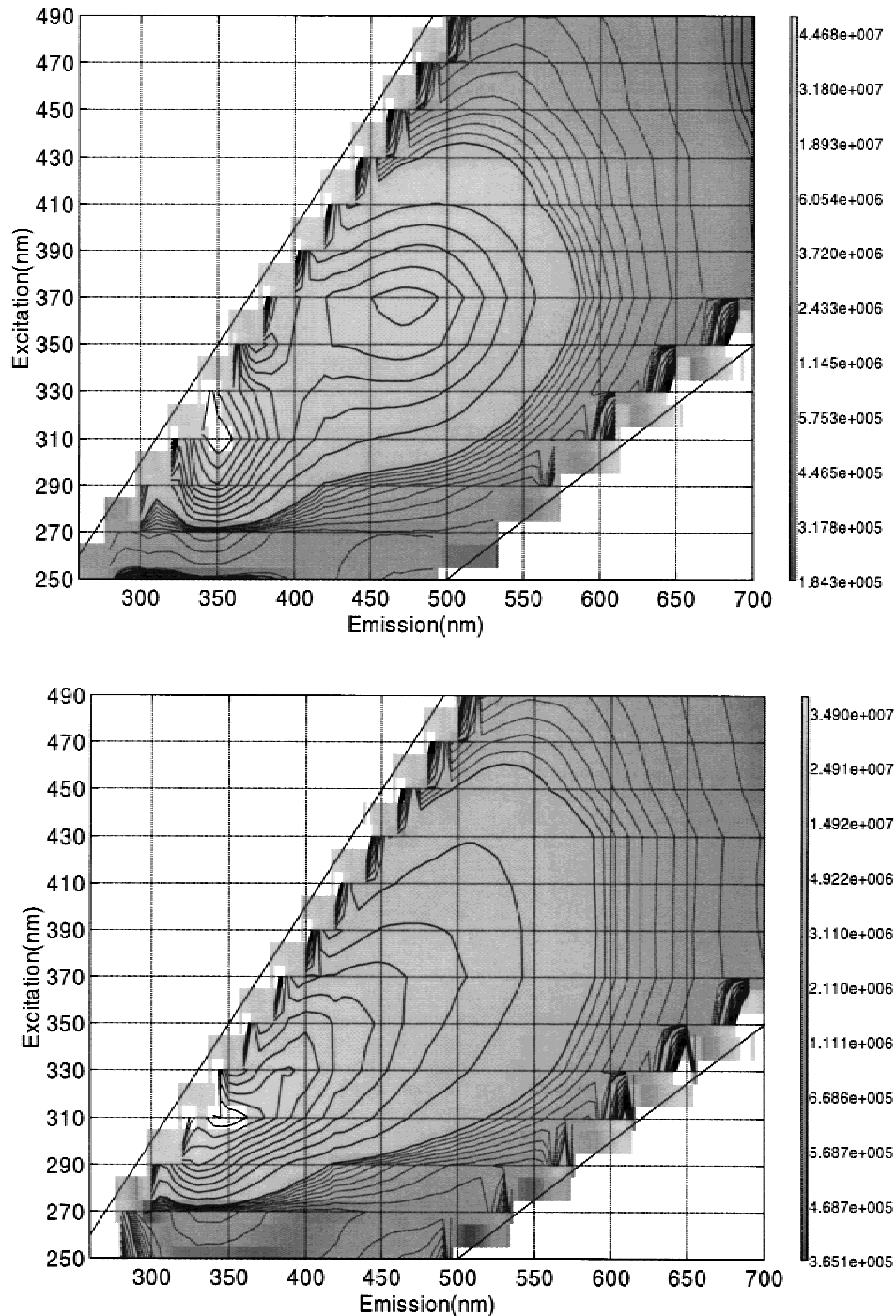


Fig. 11. EEMs of Premarin (upper) and Monistat 7 (lower).

contributed to the large variance seen in the ratio values.

Cervical Mucus

Mucus appears to be devoid of any distinct absorption bands which might significantly dis-

tort the fluorescence spectra measured from the cervix above 300 nm. However, the transmission does monotonically decrease as the wavelength decreases. For one of the mucus samples, as little as 40% of the excitation light penetrates at 337 nm, the minimum excitation wavelength used by

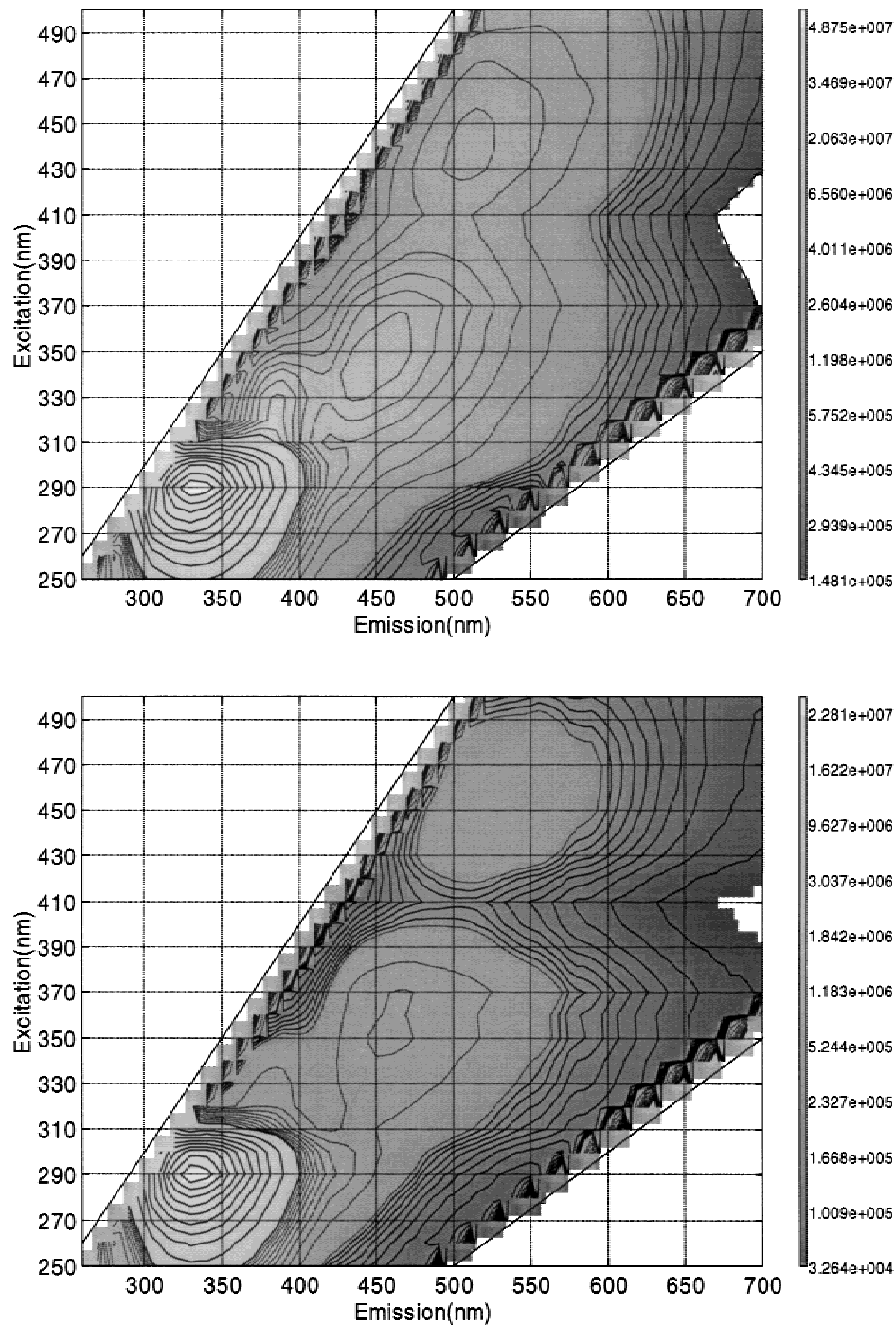


Fig. 12. EEMs of normal (upper) and abnormal (lower) cervix biopsies.

our group when acquiring fluorescence spectra for diagnostic purposes. Still, the significance of the absolute transmission values must be carefully considered, because the measurements were performed on diluted mucus samples ($\approx 7:1$ dilution factor) with a thickness (1 cm) much larger than what would be encountered on the tissue. Consid-

ering Beer's Law, which indicates that the optical density of a substance is directly proportional to both the concentration and thickness, the measurements performed in this study would be equivalent to those performed on undiluted samples with a thickness greater than 1 mm. This is a much larger thickness than what might be

encountered under a probe placed on the tissue with some pressure. Additionally, acetic acid is used at colposcopy to help remove mucus. In any case, the presence of mucus can somewhat affect the signal-to-noise ratio of the data, especially at the shorter wavelengths.

In a previous study [10], fluorescence EEMs were measured from normal and abnormal cervical biopsies *in vitro*. The same instrumentation and geometry were used to measure these EEMs as for the cervical mucus and medications here; therefore, the arbitrary intensity units are comparable to those of the EEMs in this study. The average EEMs for normal and abnormal cervical tissue are shown in Figure 12, and their excitation-emission peaks are listed in Table 2. These EEMs were compared to the EEMs collected in this study by examining the strength of the fluorescence signal and the location of excitation-emission peaks.

Comparing the peak intensities of the fluorescence EEMs, the mucus fluorescence is approximately the same order of magnitude as that of the cervical biopsies at the tryptophan peak (290/330), an NADH peak (350/450), and an FAD peak (450/535). Again considering the dilution factor and thickness of the mucus samples for these fluorescence measurements, this indicates that mucus present on the cervix can contribute to the measured fluorescence. Thus, the clinician should make all efforts to remove as much mucus as possible from the cervix before fluorescence measurements to minimize any possible contaminating absorption or fluorescence effects.

Vaginal Medications

Comparing the peak intensities of the cervical biopsies to those of the medications, it is clear that many of the medications fluoresce significantly and thus their spectral characteristics must be considered. From the database of tissue data collected in the previous *in vivo* studies, we know that the peak emission wavelengths are 440–460 nm, 460–480 nm, and 515–525 nm for excitation at 337, 380, and 460 nm respectively [8]. For 15 of the 16 medications, the peak emission wavelengths at 337 nm excitation are not in the region of diagnostic interest, that is 440–460 nm. Rather, all but two medications peak below 400 nm and one peaks at about 550 nm. However, the peaks in the emission spectra for 380 and 460 nm excitation overlap much more with the spectral region that is diagnostically useful.

Because of their significant fluorescence, the presence of any medications must be considered when measuring the fluorescence of the cervix. A simple solution to this problem is to instruct patients not to use any vaginal medication for two days prior to their Pap smear or fluorescence examination. In addition, medical providers can be instructed to remove any visible medication from the cervix with saline or acetic acid before tissue fluorescence is collected. However, the fluorescence data used in this analysis were obtained from the medications themselves, rather than from the medications on the cervix. These medications are metabolized *in vivo*, and we did not address how this affects their fluorescence or that of the cervix.

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